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Escin, a Pentacyclic Triterpene, Chemosensitizes Human Tumor Cells through Inhibition of NF- κ B Signaling Pathway

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Running title: Chemosensitization of Tumor cells by Escin

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Abbreviations used: COX-2, cyclooxygenase-2; EMSA, electrophoretic mobility-shift assay; HIV-1, human immunodeficiency virus-1; IAP, inhibitor of apoptosis protein; ICAM-1, intercellular adhesion molecule; IKK, inhibitory κ B kinase; MMP-9, matrix metalloproteinase-9; NF- κ B, nuclear factor-kappaB; NIK, NF- κ B-inducing kinase; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; SEAP, secretory alkaline phosphatase; TNF, tumor necrosis factor; TNFR1, TNF receptor; TRADD; TNFR1-associated death domain; TRAF2, TNFR-associated factor-2; VEGF, vascular endothelial growth factor

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Abstract

Agents that can enhance tumor cell apoptosis and inhibit invasion have potential for treatment of cancer. Here, we report the identification of escin, a pentacyclic triterpenoid from horse chestnut that exhibits antitumor potential against leukemia and multiple myeloma. Whether examined by esterase staining, phosphatidyl-serine staining, DNA breakage, or caspase mediated PARP cleavage, escin potentiated TNF-induced apoptosis but inhibited tumor cell invasion. This correlated with the down-regulation of bcl-2, IAP2, cyclin D1, COX2, ICAM-1, MMP-9 and VEGF, all regulated by the activation of the transcription factor NF- κ B. When examined by electrophoretic mobility shift assay, the triterpenoid suppressed NF- κ B activation induced by TNF and other inflammatory agents and this correlated with inhibition of I κ B α phosphorylation and degradation, inhibition of IKK activation, suppression of p65 phosphorylation and nuclear translocation and abrogation of NF- κ B-dependent reporter activity. Overall our results demonstrate that escin inhibits activation of NF- κ B through inhibition of IKK, leading to downregulation of NF- κ B-regulated cell survival and metastatic gene products and thus resulting in sensitization of cells to cytokines and chemotherapeutic agents.

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Introduction

Traditional medicine (TM), while in use for thousands of years, lacks an established molecular basis as defined within the last half a century. Identification of the active component and its mechanism of action can make TM the equivalent to modern medicine. For instance the extracts from the seeds of horse chestnut (*Aesculus hippocastanum*) have been traditionally used in China as carminative, stomachic and analgesic agent and an anti-fever, and anti-hemorrhoidal agent (Matsuda et al., 1997). The saponin mixture isolated from the seeds is a pentacyclic triterpene and is referred to as escin which exists in alpha and beta form (see Fig. 1A). It is the beta form of escin that has been described to exhibit ant inflammatory (Matsuda et al., 1997; Rothkopf and Vogel, 1976), anti-edema, capillary protective, hypoglycemic (Kimura et al., 2006) , anti-obesity (Hu et al., 2008) and ethanol absorption inhibitory (Sirtori, 2001; Yoshikawa et al., 1996) activities. Escin was found to inhibit acute inflammation induced by acetic acid in mice and histamine in rats (Matsuda et al., 1998) , suppress traumatic brain injury in rats (Xiao and Wei, 2005), attenuate postoperative adhesions (Fu et al., 2005), accelerate gastrointestinal transit (Matsuda et al., 1999), inhibit brain ischemia injury-induced apoptosis in rats (Hu et al., 2004), abrogate ovariectomized induced osteopenia in rats (Pytlik et al., 2000; Pytlik et al., 1999), manifest hypoglycemic activity (Yoshikawa et al., 1996) and exhibit anti-ulcerogenic effects (Marhuenda et al., 1994). This triterpene was also found to inhibit chronic aberrant foci formation in rats, and induce apoptosis in human colon cancer HT29 cells (Patlolla et al., 2006). Escin is in clinical trial in patients with HIV-1 (Grases et al., 2004) for treatment of blunt impact injuries (Wetzel et al., 2002), and for cutaneous pruritus (Li et al., 2004). How escin mediates

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all these effects, is not fully understood but suppression of inflammation has been suggested in most cases. This triterpene was found to suppress the expression of adhesion molecules on endothelial cells (Hu et al., 2004; Montopoli et al., 2007), prevent hypoxia-induced adhesiveness of neutrophils to endothelial cells (Arnould et al., 1996), and inhibit HIV-1 protease (Yang et al., 1999).

How this triterpene mediates its effect, is not well understood. Since several of these activities have been linked with the activation of NF- κ B, we postulated that escin must manifest its effects through the suppression of the NF- κ B pathway. We described here the evidence that escin can inhibit the activation of NF- κ B induced by various cytokines and carcinogenic stimuli. This leads to the downregulation of NF- κ B-linked gene products, potentiation of apoptosis and inhibition of invasion.

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Materials and Methods

Reagents: A 50 mM solution of escin (Sigma-Aldrich, USA) was prepared initially in DMSO, stored as small aliquots at -20°C, and then thawed and diluted in a cell culture medium as required. Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640, IMDM, and DMEM were obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was supplied by Atlanta Biological (Lawrenceville, GA). Antibodies against p65, p50, I κ B α , cyclin D1, cyclooxygenase-2 (COX-2), matrix metalloproteinase (MMP)-9, poly (ADP-ribose) polymerase (PARP), inhibitor of apoptosis protein (IAP)-2, bcl-2, and intercellular adhesion molecule (ICAM)-1, c-Jun kinase (JNK)-1, p38 MAPK, p44/42 MAPK (ERK1/2), TNF-receptor (TNFR)-1, TNFR1-associated death domain (TRADD), TNFR-associated factor (TRAF)-2, NF- κ B inducing kinase (NIK), and the Annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For immunocytochemistry, an antibody against p65 was obtained from Abcam (Cambridge, MA). An anti-vascular endothelial growth factor (VEGF) antibody was purchased from Thermo Scientific (Fremont, CA). Phospho-specific anti-I κ B α (Ser32/36) and phosphor-specific anti-p65 (Ser536) antibodies were purchased from Cell Signaling Technology. Anti-IKK- α , anti-IKK- β , and IKK- γ antibodies were provided by Imgenex (San Diego, CA).

Cell lines: The cell lines KBM-5 (human chronic myeloid leukemia), A293 (human embryonic kidney carcinoma), H1299 (human lung adenocarcinoma), Jurkat (Human T-cell leukemia) and U266 (human multiple myeloma) were obtained from the American

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Type Culture Collection. KBM-5 cells were cultured in IMDM with 15% FBS; H1299, Jurkat and U266 cells were cultured in RPMI 1640 supplemented with 10%FBS; and A293 cells were cultured in DMEM supplemented with 10% FBS. All culture media were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin.

Electrophoretic mobility shift assay: To assess effects of escin on NF-κB activation, electrophoretic mobility shift assay (EMSA) was performed as described previously (Chaturvedi et al., 2000). Briefly, nuclear extracts prepared from treated cells were incubated with ³²P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 µg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'-TTGTTACAA **GGGACTTTCCGCTG GGGACTTTCCAGGGAGGCGTGG**-3' (boldface indicates NF-κB-binding sites), for 30 min at 37°C, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACA**CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3', was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum (PIS) was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantitated using ImageQuant software (GE Healthcare, Piscataway, NJ).

Immunoprecipitation (IP) and Western blot analysis: To determine the levels of protein expression, we prepared either cytosolic or nuclear or whole cell extracts (Sethi

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et al., 2007) and fractionated them by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electro transferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by ECL reagent (GE Healthcare).

To see the effects of escin on ubiquitination of IKK- γ induced by TNF, IP and western blot were performed. Whole cell extract were prepared from treated cells. Following antibodies were used for IP and IB: anti-IKK- γ antibody (IP: 1:100; IB:1:1000, Imgenex), anti-ubiquitin antibody (IB: 1:1000, Santa Cruz). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKK- γ and then treated with protein A/G-agarose beads (Pierce, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then resuspended in a lysis buffer and boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS–PAGE and transferred to a nitrocellulose membrane and then blotted with either anti-ubiquitin or anti-IKK- γ antibody.

Kinase assay: To determine the effect of escin on TNF-induced IKK activation, IKK assay was performed by a method we described previously (Sethi et al., 2007), with the following exceptions. Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKK- β and then treated with protein A/G-agarose beads. After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM MgCl₂, 2 mM DTT, 20 μ Ci [γ -³²P]ATP, 10 mM unlabeled ATP, and 2 μ g of substrate GST-I κ B α (aa1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS–PAGE, the gel was

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dried, and the radioactive bands were visualized with a Storm 820. To determine the total amounts of IKK- α and IKK- β in each sample, 30 μ g of whole-cell proteins was resolved on 7.5% SDS-PAGE, electro transferred to a nitrocellulose membrane, and then blotted with either anti-IKK- α or anti-IKK- β antibody.

For JNK assay, whole cell extracts were precipitated with antibody against JNK1, and performed kinase assay using GST-c-Jun (aa 1–79). To determine the amount of JNK1 in each sample, Western blotting was performed against JNK1 antibody.

Immunocytochemical analysis for NF- κ B p65 localization: The effect of escin on TNF-induced nuclear translocation of p65 was examined using an immunocytochemical method. Briefly, KBM-5 cells were initially seeded in a 12 well plates. The cells were treated with escin for 2 h followed by stimulation with 0.1 nM TNF for 15 min. After cytospin slides were air-dried and fixed with 4% paraformaldehyde followed by permeabilization with 0.2% of Triton X-100. After being washed in PBS, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal antihuman p65 antibody at a 1:100 dilution followed by overnight incubation at 4 °C. The slides were again washed in PBS, incubated with goat anti-rabbit IgG-Alexa 594 at a 1:200 dilution for 1 h. The nuclei were counterstained with Hoechst 33342 (50 ng/ml) for 5 min. The stained slides were mounted with a mounting medium (Sigma-Aldrich) and cells were visualized under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

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NF- κ B-dependent reporter gene expression assay: The effects of escin on NF- κ B-dependent reporter gene transcription induced by TNF was analysed by secretory alkaline phosphatase (SEAP) assay as previously described (Darnay et al., 1998), with the following exceptions. Briefly, A293 cells (5×10^5 per well) were plated in six-well plates and transiently co-transfected with 0.5 μ g of reporter vector pNF- κ B-SEAP and 1.5 μ g of the control plasmid (pCMV-FLAG1) by the calcium phosphate method for 24 h. After 24 h of transfection, cells were treated with Escin for 2 h and stimulated with 1 nM TNF for 24 h. After 24 h of TNF treatment the cell culture medium was harvested and SEAP assay was performed.

To determine reporter gene expression induced by various genes, A293 cells were transiently co-transfected with 0.5 μ g of pNF- κ B-SEAP plasmid along with 0.5 μ g of an expressing plasmid (TNFR1, TRADD, TRAF2, NIK, IKK β and p65) and 1.0 μ g of control plasmid (pCMV-FLAG1) for 24 h. After 24 h of transfections, cells were treated with or without escin for 2 h. The cell culture medium was harvested from the cells after an additional 24 h of incubation. The culture medium was analyzed for SEAP activity using a Victor 3 microplate reader (Perkin-Elmer Life Sciences). Cells were also harvested and whole cell lysates were prepared. The expressions of vectors were assessed by western blot with relevant antibodies.

Live/Dead assay: To measure apoptosis, we performed Live/Dead cell viability assay (Invitrogen), which is an index of intracellular esterase activity and plasma membrane integrity. One of the components of the kit is a nonfluorescent polyanionic dye (calcein-AM) which is retained by live cells and it produces intense green fluorescence through enzymatic (esterase) conversion. In addition, the ethidium homodimer enters cells

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through damaged membranes and binds to nucleic acids, thereby producing a bright red fluorescence in dead cells. Briefly, 2×10^5 cells were plated in 12 well plates and incubated with 30 μ M escin for 2 h and treated with 1 nM TNF up to 16 h at 37°C. Cells were stained with the Live/Dead reagent (5 μ M ethidium homodimer and 5 μ M calcein-AM) and incubated further at 37 °C for 30 min. Cells were visualized under a fluorescence microscope (Labophot-2; Nikon).

Annexin V assay: To identify phosphatidylserine externalization during apoptosis, cells were stained with an Annexin V antibody conjugated with the fluorescent dye FITC. In brief, 5×10^5 cells were co-incubated with 30 μ M escin and 1 nM TNF for 16 h, stained with annexin V-FITC conjugate, and then analyzed using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling: To measure the DNA strand breaks during apoptosis, we performed the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, which employs the *in situ* Cell Death Detection reagent (Roche Molecular Biochemical). In brief, 5×10^5 cells were co-incubated with 30 μ M escin and 1 nM TNF for 16 h and then incubated with a reaction mixture. Stained cells were analyzed using a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA).

Invasion assay: Invasion through the extracellular matrix is a crucial step in tumor cell metastasis. To determine the effect of escin on TNF-induced invasion we used the BD BioCoat tumor invasion system (BD Biosciences) and followed protocol was similar to that described earlier (Ahn et al., 2007). Briefly, H1299 cells (2.5×10^4 cells) were resuspended in serum-free medium and seeded into the upper wells. After incubation

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overnight, the cells were treated with 30 μ M escin for 2 h and then stimulated with 1 nM TNF for 24 h in the presence of 1% FBS . After incubation, the cells were stained with 4 μ g/mL calcein acetoxymethylester (Invitrogen) in PBS for 30 min at 37 °C to detect the invaded cells through the Matrigel. The fluorescence intensity was measured with a Victor 3 (PerkinElmer Life and Analytical Sciences).

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Results

The goal of the present study was to determine whether escin modulates NF- κ B-mediated signal transduction, cellular response and NF- κ B-regulated gene expression. We conducted most of our studies using KBM-5 cells with TNF as an inducer of biological response. We used KBM-5 cells because these are known to have both types of TNF receptors. We focused on TNF-induced NF- κ B activation because the NF- κ B activation pathway activated by TNF has been relatively well characterized.

Escin potentiates TNF-induced apoptosis in leukemia and myeloma cells: To determine whether escin modulates TNF-induced apoptosis in KBM-5 and Jurkat cells, we performed the Live/Dead, annexin V-FITC, TUNEL and PARP cleavage assay. We first determined the optimum dose of escin required potentiating apoptotic effects of TNF (Fig. 1B). We found that 30 μ M of escin is optimum to induced apoptotic effects of TNF. We then, assessed apoptotic effects of escin through live/dead assay. We found that escin increased the TNF-induced apoptosis in both type of cells, from 7% to 60% and 6% to 70% in KBM-5 and Jurkat cells, respectively (Fig. 1C, left and right panel).

To further demonstrate that escin can potentiate TNF-induced apoptosis, we used the annexin V method. The result shown in Fig.1D revealed that TNF-induced apoptosis was enhanced in the presence of escin (left panel). Moreover, results of TUNEL staining assay also strengthen that TNF-induced apoptosis was enhanced in presence of escin (Fig. 1D, right panel). Escin also potentiated the TNF-induced caspase-mediated PARP cleavage, (Fig. 1E). Overall these results show that escin potentiated the apoptotic activity of TNF.

Escin suppresses TNF-induced tumor cell invasion: We also analyzed whether

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escin can modulate TNF-induced tumor cell invasion *in vitro* using a matrigel invasion assay. As shown in Fig. 1F, escin inhibited the TNF-induced invasion of tumor cells, indicating its anti-invasive potential.

Escin inhibits TNF-induced cell survival gene products: How escin potentiates the apoptotic effects of TNF were investigated by examining the expression of the cell survival gene products bcl-2 and IAP. We found that TNF-induced the expression of bcl-2 and cIAP-2 and escin inhibited the expression (Fig. 2A).

Escin suppresses the TNF-induced expression of cell-proliferative gene products: Both cyclin D1 and COX-2 have been linked with the proliferation of different types of tumor cells. Thus, we investigated whether escin affected the expression of cyclin D1 and COX-2 induced by TNF treatment. We found that TNF induced the expression of these gene products and that treatment with escin inhibited this expression (Fig. 2B).

Escin suppresses the TNF-induced expression of gene products involved in invasion and angiogenesis: TNF also induces the expression of genes involved in invasion such as MMP-9 and ICAM-1. We examined the effect of escin on TNF-induced MMP-9 and ICAM-1 expression in KBM-5 cells. We found that escin inhibited the expression of both the gene products (Fig. 2C). VEGF is involved in the process of tumor angiogenesis. We found that TNF-induced expression of VEGF and escin inhibited the expression (Fig. 2C).

Escin inhibits TNF-induced NF- κ B activation: We found that escin inhibits levels of bcl-2, IAP-2, COX-2, cyclin D1, MMP-9, ICAM-1 and VEGF induced by TNF. How escin inhibits the expression of all these various gene products was investigated. Because all above said genes products are regulated by the NF- κ B, we examined the effect of escin

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on the NF- κ B pathway. We pretreated KBM-5 cells with various concentrations of escin for 2 h and then, cells were stimulated with TNF for the activation of NF- κ B. As results indicate that escin by itself had no effect on the activation of NF- κ B. However, escin suppressed the TNF-induced NF- κ B activation in a dose-dependent manner, with maximum inhibition occurring at 30 μ M (Fig. 3A). Cells viability under these conditions was greater than 90%.

We then exposed KBM-5 cells to escin for different time intervals followed by treatment with TNF. We found that escin suppressed the activation of NF- κ B induced by TNF in a time-dependent manner, with optimum inhibition occurring at 2 h (Fig. 3A, right panel).

NF- κ B is a complex of proteins, in which various combinations of Rel/NF- κ B proteins constitute active NF- κ B heterodimers that bind to a specific DNA sequence. Thus, to show that the band visualized by EMSA in TNF-treated cells was indeed NF- κ B, nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF- κ B) and p65 (RelA) subunits of NF- κ B and analyzed by EMSA. The results in Fig. 3B showed the bands had shifted to higher molecular masses suggesting that the TNF-activated complex consisted of p50 and p65. Pre-immune serum (PIS) had no effect on DNA binding. The addition of excess unlabeled NF- κ B (cold oligonucleotide; 100-fold) caused a complete disappearance of the band, whereas the addition of mutated oligonucleotide had no effect on the DNA binding.

Escin does not directly modulate the binding of NF- κ B p65 subunit to the DNA:

We also checked whether escin can directly interact with the p65 subunit of NF- κ B and abolish its binding to DNA. Nuclear extracts isolated from TNF-treated KBM-5 cells were

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exposed to escin at different concentrations and then examined for binding to DNA. We found that escin did not interfere with the p65 binding to DNA (Fig. 3C).

Inhibition of NF- κ B activation by escin is not cell type-specific: Whether the inhibition of NF- κ B by escin is specific to a particular cell type was examined. Human lung adenocarcinoma (H1299) cell were pretreated with different concentrations of escin and stimulated with TNF. EMSA showed that escin inhibited the activation of NF- κ B in a dose dependent manner (Fig. 3D, left panel).

Several tumor cell types are known to constitutively express NF- κ B. Multiple myeloma cells (U266) in particular are known to have constitutively active NF- κ B. To determine whether escin affects NF- κ B expression, we exposed U266 cells to escin at different concentrations for 2 h and then analyzed them for DNA binding. Escin completely suppressed constitutive NF- κ B activation in U266 cells (Fig. 3D, right panel), indicating that this triterpene can suppress both inducible and constitutive NF- κ B activation and the effects are not cell type specific.

Escin inhibits NF- κ B activation induced by carcinogens and other inflammatory stimuli: Studies reported from our laboratory and by others showed that a wide variety of agents including cigarette smoke condensate (CSC), tumor promoters (e.g., okadaic acid [OA], Phorbol myristate acetate [PMA]), inflammatory agents such as hydrogen peroxide (H₂O₂) and lipopolysaccharide [LPS], can activate NF- κ B but the mechanisms by which these agents induce activation of NF- κ B vary significantly. We examined whether escin affects NF- κ B activation induced by H₂O₂, PMA, LPS, OA and CSC. We found that all of these agents activated NF- κ B in KBM-5 cells and that escin suppressed

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this activation (Fig. 3E). Therefore, we concluded that this triterpene acts at a step in the NF- κ B activation pathway that is common to all of these agents.

Escin inhibits TNF-dependent phosphorylation and degradation of I κ B α : I κ B α is the inhibitory subunit associated with the NF- κ B complex. Translocation of NF- κ B to the nucleus is accompanied by phosphorylation, ubiquitination, and degradation of I κ B α . To determine whether inhibition of TNF-induced NF- κ B activation is associated with degradation of I κ B α , we pretreated KBM-5 cells with escin and then exposed them to TNF at various time points. We analyzed nuclear extracts for NF- κ B activation using EMSA and cytoplasmic extracts for I κ B α degradation using Western blotting. As shown by EMSA, TNF activated NF- κ B in a time-dependent manner; however, we observed no activation of NF- κ B in escin-pretreated cells (Fig. 4A, upper panel).

Analysis of cytoplasmic extract, through western blot, showed that TNF-induced I κ B α degradation started at 10 min after TNF treatment and reached maximum level at 30 min, and that resynthesis occurred at 60 min after TNF treatment (Fig. 4A, lower panel). However, we noticed that no degradation of I κ B α in escin-pretreated cells (Fig. 4A, lower panel). These results indicate that escin mediates its effect *via* suppression of TNF-induced I κ B α degradation, which eventually leads to suppression of NF- κ B activation.

To determine whether inhibition of TNF-induced degradation of I κ B α was caused by inhibition of phosphorylation of I κ B α , we used the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) to block this degradation. Western blot analysis was done using an antibody that specifically recognized the I κ B α phosphorylated at serine

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32/36. The results of this analysis showed that TNF induced I κ B α phosphorylation at serine 32/36 and that escin strongly inhibited this phosphorylation (Fig. 4B).

Escin inhibits TNF-induced IKK- γ (NEMO) ubiquitination: The IKK signalosome contains the kinase, IKK- α and/or IKK- β , and the highly conserved regulatory protein NEMO (also known as IKK- γ or FIP-3). IKK activity relies on the interaction between the kinase and NEMO (Scheidereit, 2006). TNF induces ubiquitination of IKK- γ , which activate IKK. We sought to determine whether escin inhibits TNF-induced ubiquitination of IKK- γ in KBM-5 cells. We first pretreated cells with proteasome inhibitor ALLN to block any degradation. After ALLN exposure cells were treated with escin and finally challenged with TNF. The results showed that TNF strongly induced ubiquitination of NEMO and that escin suppressed (Fig. 4C).

Escin inhibits TNF-induced I κ B α kinase (IKK) activation: TNF-induced phosphorylation of I κ B α requires activation of the enzyme IKK. We sought to determine whether escin inhibits TNF-induced activation of IKK in KBM-5 cells using immune complex assays. These assays showed that TNF activated IKK in a time-dependent manner and that the triterpene suppressed TNF-induced activation of IKK. Neither TNF nor triterpene affected the expression of IKK- α or IKK- β protein (Fig. 4D).

Our next aim was to determine whether escin suppresses IKK activity directly by binding to IKK or indirectly by suppressing its activation. We first prepared TNF-induced whole cell lysate. Portion of the whole cell lysate was pool down with IKK- β . Final, immune complex was incubated with various concentrations of escin and then examined for the IKK activity *in vitro*. The results showed that escin did not directly inhibit activity of IKK (Fig. 4E).

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Whether escin mediates its effect on TNF-induced IKK activation through reactive oxygen species (ROS), was determined by using ROS quencher N-acetyl cysteine (NAC). We now demonstrate that TNF activated IKK and quenching of ROS by NAC, only partially suppresses IKK activation (Fig. 4F). We also noted that suppression of IKK by escin is not reversed by NAC. These results suggest that it is unlikely that the effects of escin on NF- κ B activation are mediated through ROS.

Escin does not inhibit TNF-induced MAPK activation: The effect of escin on TNF-induced MAPK activation pathway was also examined. The results in Fig. 4G indicated that TNF-induced p38 MAPK and p44/42 MAPK activation, however escin had no inhibitory effect on the activation of these kinases. When examined for c-Jun kinase, we found escin alone activated the kinase to the same extent as TNF itself (Fig. 4G).

Escin inhibits TNF-induced translocation of NF- κ B p65 subunit: The degradation of the inhibitory subunit present in the NF- κ B complex (I κ B α) initiates the nuclear translocation of p65. We sought to determine whether escin has any effect on TNF-induced nuclear translocation of p65. Immunocytochemical analysis showed that escin suppressed the TNF-induced translocation of p65 to the nucleus in KBM-5 cells (Fig. 5A). In both untreated cells and cells treated with escin, p65 was localized to the cytoplasm, whereas in cells treated with TNF alone, p65 was translocated to the nucleus. These results support the notion that that escin inhibits translocation of p65.

We further evaluated the TNF-induced translocation by western blot. We found that TNF induced nuclear translocation of p65 in a time-dependent manner, and pretreatment with escin blocked its translocation (Fig. 5B).

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Escin inhibits TNF-induced phosphorylation of NF- κ B p65 subunit: We also investigated the effect of escin on the TNF-induced phosphorylation of p65 at serine residue 536, because phosphorylation is required for the transcriptional activity of p65. TNF-induced p65 phosphorylation in the cytoplasm in a time-dependent manner. p65 was phosphorylated as early as 10 min after TNF stimulation and increased up to 30 min (Fig. 5B, upper panel). In cells treated with escin, TNF failed to induce p65 phosphorylation. Similar results were obtained with nuclear p65 phosphorylation (Fig. 5B, lower panel).

Escin suppresses TNF-induced, NF- κ B-dependent reporter gene expression:

Although we showed using EMSA, that escin inhibited TNF-induced NF- κ B expression, DNA binding alone does not always correlate with NF- κ B-dependent gene transcription, suggesting that additional regulatory steps are involved. Thus, we also determined whether escin affects TNF-induced reporter gene transcription. For this, cells were transiently transfected with an NF- κ B-regulated SEAP reporter construct (pNF- κ B SEAP), treated with escin, and then exposed to TNF. We found that TNF induced NF- κ B reporter activity and escin inhibited the TNF-induced NF- κ B reporter activity in a dose-dependent manner (Fig. 6A).

Escin inhibits NF- κ B activation stimulated by TNFR1, TRADD, TRAF, NIK, IKK and

p65: TNF-induced NF- κ B activation requires a sequential recruitment of TNFR1, TRADD, TRAF2, NIK and IKK- β . To determine where in the pathway escin blocks the TNF-induced NF- κ B activation, we decided to examine the effect of escin on TNFR1, TRADD, TRAF2, NIK, IKK- β and p65-induced NF- κ B-dependent reporter gene

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transcription. The results presented in Fig. 6B show that all of these plasmids induced NF- κ B reporter activity and that escin inhibited the activation.

To verify that the expression vectors used indeed produce the predicted protein in the transfected cells, whole cell extracts were prepared and analyzed by Western blot using antibodies against TNFR1, TRADD, TRAF2, NIK, IKK- β , and p65. It was observed that these plasmids indeed enhanced the expression of the predicted proteins over the basal levels in cells (Fig. 6C).

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Discussion

Our present study was designed to identify the molecular basis for various pro-inflammatory responses modulated by escin. We examined the effect of this triterpene on NF- κ B activation induced by various inflammatory stimuli. DNA binding assays indicated that escin inhibited the NF- κ B activation induced by TNF, H₂O₂, PMA, LPS, OA and CSC. The inhibition of NF- κ B activation induced by all these agents suggests that escin must act at a step that is common to all these agents. Xiao and Wei showed that trauma-induced NF- κ B activation is blocked in the brain of rats treated with escin (Xiao and Wei, 2005). Although no mechanism by which escin inhibits NF- κ B activation was provided, our results on NF- κ B inhibition are consistent with this report. Besides inducible activation, we also found that escin abolished the constitutive NF- κ B activation expressed by most tumor cell types.

How escin inhibits NF- κ B activation was also investigated for the first time in detail. We found that the triterpenoid inhibited the phosphorylation and degradation of I κ B α . Since phosphorylation of I κ B α is catalyzed by I κ B α kinase (IKK), we found that escin inhibits the activation of IKK through inhibition of ubiquitination of IKK- γ . Further investigation showed that escin did not directly inhibit the activity of IKK but blocked the activation of this kinase. Numerous kinases have been linked with the activation of IKK (Hacker and Karin, 2006) While NIK has been linked with activation of IKK by CD40L and receptor activator for NF- κ B ligand (RANKL), TAK1 has been linked with the activation of IKK by TNF (Jackson-Bernitsas et al., 2007). We found that escin blocked the NF- κ B activation induced by both NIK and IKK. IKK has been linked with the phosphorylation of p65 and we found that TNF-induced phosphorylation of p65, was

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also blocked by escin.

NF- κ B activation has been shown to cause the expression of several gene products involved in the process of cell survival, proliferation, apoptosis, invasion, metastasis and angiogenesis (Aggarwal, 2004). We found escin suppressed the TNF-induced expression of bcl-2, IAP2, cyclin D1, COX-2, ICAM-1, MMP-9 and VEGF. Previous reports shown that this triterpene could suppress the expression of adhesion molecules on endothelial cells (Hu et al., 2004; Montopoli et al., 2007) and prevents hypoxia-induced adhesiveness of neutrophils to endothelial cells (Arnould et al., 1996) are consistent with suppression of ICAM-1 expression shown here. There is no previous report about the effect of escin on the modulation of other gene products as described here. It is possible, however, that several of the activities previously assigned to escin are due to modulation of these gene products. These include anti-inflammatory (Matsuda et al., 1997), anti-edema, capillary protective, hypoglycemic (Kimura et al., 2006), and anti-obesity (Hu et al., 2008) activities. Inhibition of acute inflammation in rats (Matsuda et al., 1997); attenuation of postoperative adhesions (Fu et al., 2005); inhibition of ovariectomy-induced osteopenia in rats (Pytlik et al., 2000; Pytlik et al., 1999); suppression of hypoglycemia (Yoshikawa et al., 1996); and inhibition of chronic aberrant foci formation in rats (Patlolla et al., 2006), could also be due to inhibition of NF- κ B by escin.

We found that escin significantly potentiated TNF-induced apoptosis, which could be due to suppression of cell survival (bcl-2 and IAP2) and proliferative (cyclin D1, and COX-2) gene products. This triterpene was also found to induce growth arrest at the G1-S phase and induce apoptosis in human colon cancer HT29 cells (Patlolla et al.,

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2006). Since cyclin D1 has been closely linked with G1-S phase arrest, downregulation of cyclin D1 as shown here may mediate this effect.

Most cancer-associated deaths are due to invasion of tumors into vital organs, which in turn due to expression of MMP-9 and VEGF. We found that this triterpinoid suppressed the expression of both of these gene products. We also showed that TNF-induced invasion of tumor cells was suppressed by the triterpene. Thus, overall these results suggest that escin has a potential in the prevention and treatment of cancer. Several studies in animals suggest that escin is very well tolerated and has potential against inflammatory diseases. Escin is currently in clinical trial and has been found to be quite safe. More studies are needed to fully appreciate its potential against various chronic inflammatory diseases. In conclusion, our results clearly demonstrated the anti-proliferative, anti-invasive and anti-inflammatory activities of escin are mediated through the inhibition of NF- κ B and NF- κ B-regulated gene products.

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Figure legends

FIGURE 1: Effects of escin on TNF-induced apoptosis.

A, The chemical structure of escin. **B**, Escin potentiates apoptotic effects TNF. KBM-5 cells (5000 cells/well) were treated with indicated amounts of escin for 2h followed by indicated amounts of TNF for 24 h at 37°C. Cell viability was assessed by MTT uptake method. **C**, KBM-5 cells (*left panel*) and Jurkat cells (*right panel*) were pretreated with 30 μ M escin for 2 h and then incubated with 1 nM TNF for 24 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described under "Materials and Methods". Data indicated as percentage proportions of apoptotic cells and was expressed as mean \pm S.E. **D**, (*Left panel*) Cells were pretreated with 30 μ M escin for 2 h and then incubated with 1 nM TNF for 16 h. The cells were incubated with a fluorescein isothiocyanate-conjugated annexin V antibody and then analyzed by flow cytometry as described under "Materials and Methods". (*Right panel*) Cells were pretreated with 30 μ M escin for 2 h and then incubated with 1 nM TNF for 16 h. The cells were stained for TUNEL positive cells and then analyzed by flow cytometry as described under "Materials and Methods". Determinations were made in triplicate. Data represent the mean of two measurements \pm S.E. **, $p < 0.05$. **E**, Effect of escin on PARP cleavage. Cells were pretreated with 30 μ M escin for 2 h and then incubated with 1 nM TNF for the indicated times. Whole cell extracts were prepared, and analyzed by Western blotting using an anti-PARP antibody. **F**, Escin suppresses TNF-induced invasion activity. H1299 cells (2.5×10^4 cells) were seeded to the top chamber of a Matrigel invasion chamber system overnight in the absence of serum and then treated with 30 μ M escin. After incubation, the cells were

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treated with TNF in the presence of 1% serum and then assayed for invasion as described under "Materials and Methods." Results are expressed as fold activity of the untreated control. All results shown are representative of two independent experiments.

FIGURE 2: Effects of escin on TNF-induced cell survival, cell-proliferative, and metastatic gene products.

A, Escin suppresses the expression of TNF-induced anti-apoptotic proteins. **B and C**, Escin inhibits the expression of TNF-induced cell proliferative and metastatic proteins. KBM-5 cells (2×10^6 cells/mL) were incubated with 30 μ M escin for 2 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. All results shown are representative of two independent experiments. Densitometric values of bands were corrected based on β -actin and were expressed relative to that of untreated cells, which was set as 1.0.

FIGURE 3: Effects of escin on constitutive and induced NF- κ B activation.

A, (*Left panel*), dose dependent effect of escin on TNF-induced NF- κ B activation. KBM-5 cells (2×10^6 cells/mL) were incubated with the indicated concentrations of escin for 2 h and treated with 0.1 nM TNF for 30 min. The nuclear extracts were assayed for NF- κ B activation by EMSA. Results are expressed as fold activity of the untreated control; (*Right panel*), time dependent effect of escin on TNF-induced NF- κ B activation. KBM-5 (2×10^6 cells/mL) cells were pre incubated with 30 μ M of escin for the indicated time points and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF- κ B activation by EMSA. Results are expressed as fold activity of the untreated control. **B**, NF- κ B induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated cells or cells treated with 0.1 nM TNF were incubated

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with the indicated antibodies, an unlabeled NF- κ B oligo probe, or a mutant oligo probe. They were then assayed for NF- κ B activation by EMSA. **C**, Effect of escin on the binding of NF- κ B to DNA. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nM TNF and incubated for 30 minutes with the indicated concentrations of escin. They were then assayed for NF- κ B activation by EMSA. All results shown are representative of two independent experiments. **D**, (*Left panel*) escin inhibits NF- κ B activation induced by TNF in H1299 cells. The H1299 cells (1×10^6 cells/mL) cells were pre incubated with indicated concentration of escin for 2 h and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF- κ B activation by EMSA. Results are expressed as fold activity of the untreated control; (*right panel*), effect of escin on constitutive NF- κ B activation. Multiple myeloma U266 cells (2×10^6 cells/mL) were incubated with the indicated concentrations of escin for 2 h; the nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA. Results are expressed as fold activity of the untreated control. **E**, Escin inhibits NF- κ B activation induced by CSC, H₂O₂, PMA, LPS, okadaic acid (OA) and TNF. KBM-5 (2×10^6 cells/mL) cells were pre incubated with 30 μ M escin for 2 h and then treated with 0.1 nM TNF for 30 min, 500 nM okadaic acid for 4 h, 250 μ M H₂O₂ for 2hr, 25 ng/mL PMA for 2 h, 10 μ g/mL LPS and 10 μ g/mL CSC, 1 h each. Nuclear extracts were analyzed for NF- κ B activation by EMSA. Results are expressed as -fold activity of the untreated control. All results shown are representative of two independent experiments. WT, wild type, MT, mutant, CV, cell viability.

FIGURE 4: Effects of escin on TNF-induced IKK activation and I κ B α degradation.

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A, Escin inhibits TNF-induced NF- κ B activation and I κ B α degradation. (*Upper panel*), KBM-5 cells (2×10^6 cells/mL) were incubated with 30 μ M escin for 2 h, treated with 0.1 nM TNF for the indicated times. Nuclear extracts prepared from treated cells were analyzed for NF- κ B activation by EMSA. Results are expressed as fold activity of the untreated control; (*lower panel*), cytoplasmic extracts prepared from treated cells were analyzed by Western blotting using antibody against anti-I κ B α . Equal protein loading was evaluated by β -actin. **B**, Effect of escin on phosphorylation of I κ B α induced by TNF. Cells were preincubated with 30 μ M escin for 2 h, incubated with 50 μ g/mL *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific I κ B α antibody. **C**, Escin inhibits ubiquitination of IKK- γ induced by TNF. KBM-5 (4×10^6 cells/mL) cells were pre incubated with 30 μ M escin for 2 h, incubated with 50 μ g/mL ALLN for 30 min, and then treated with 0.1 nM TNF for 10 min. Whole cell extracts were prepared and immunoprecipitated with IKK- γ antibody. Immunoblot was performed against ubiquitin antibody and loading was confirmed by IKK- γ antibody. **D**, Escin inhibits IKK activation induced by TNF. KBM-5 (4×10^6 cells/mL) cells were pretreated with 30 μ M escin for 2 h, and then challenged with 1nM TNF for indicated time points. Whole cell extracts were immunoprecipitated with antibody against IKK- β and analyzed by an immune complex kinase assay. To examine the effect of escin on the level of expression of IKK proteins, whole cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK- α and anti-IKK- β antibodies. **E**, Direct effect of escin on IKK activation induced by TNF.

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Whole cell extracts were prepared from KBM-5 cells treated with 1 nM TNF and immunoprecipitated with anti-IKK- β antibody. The immunocomplex kinase assay was performed in the absence or presence of the indicated concentration of escin. All results shown are representative of two independent experiments. **E**, Role of ROS on escin suppressed IKK activation by TNF. KBM-5 (4×10^6 cells/mL) cells were preincubated with 10 mM N-acetyl cysteine (NAC) for 1 h, 30 μ M escin for 2 h, and then challenged with 1nM TNF for 10 min. Whole cell extracts were immunoprecipitated with antibody against IKK- β and analyzed by an immunocomplex kinase assay. **G**, Effect of escin on TNF-induced MAPK activation. Cells were preincubated with 30 μ M escin for 2 h and then treated with 0.1 nM TNF for 10 min. Whole cell extracts were fractionated and then subjected to Western blot analysis using the relevant antibodies. For JNK activation, whole cell extracts were immunoprecipitated with antibody against JNK1 and analyzed by an immunocomplex kinase assay. To examine the effect of escin on the level of expression of JNK protein, whole cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-JNK1 antibody.

FIGURE 5: Effects of escin on phosphorylation and nuclear translocation of p65 induced by TNF.

A, Escin inhibits TNF-induced nuclear translocation of p65. KBM-5 cells were first treated with 30 μ M escin for 2 h at 37 °C and then exposed to 0.1 nM TNF for 15 min. After cytospin, immunocytochemical analysis was done as described under "Materials and Methods". **B**, Escin inhibits TNF-induced translocation and phosphorylation of p65. KBM-5 cells were either untreated or pretreated with 30 μ M escin for 2 h at 37 °C and then treated with 0.1 nM TNF for the indicated times. Cytoplasmic and nuclear extracts

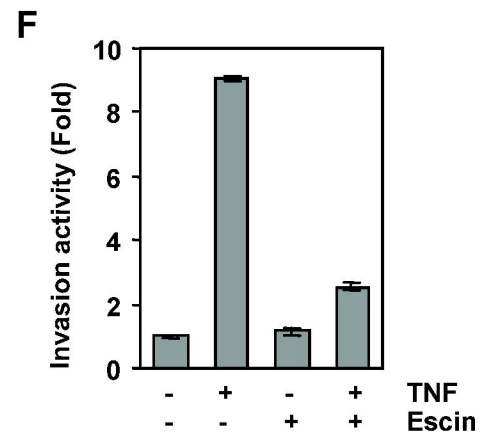
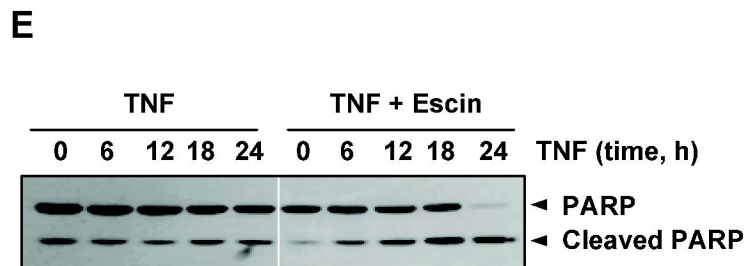
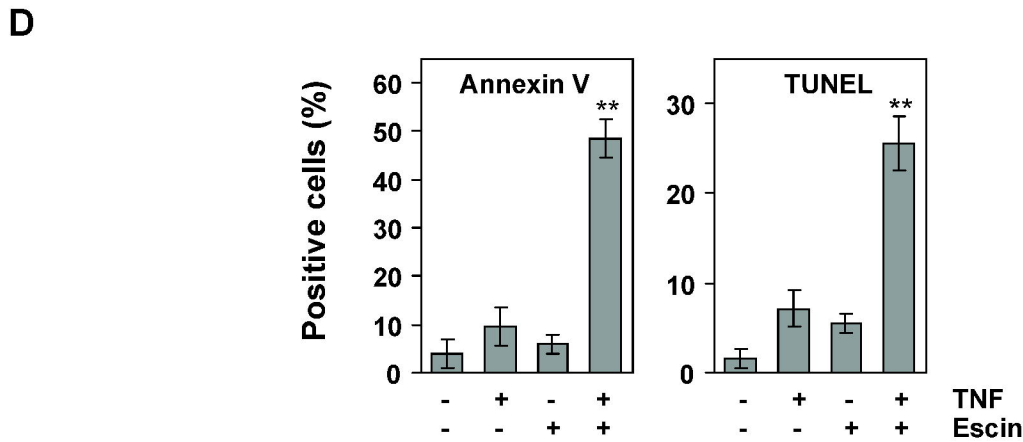
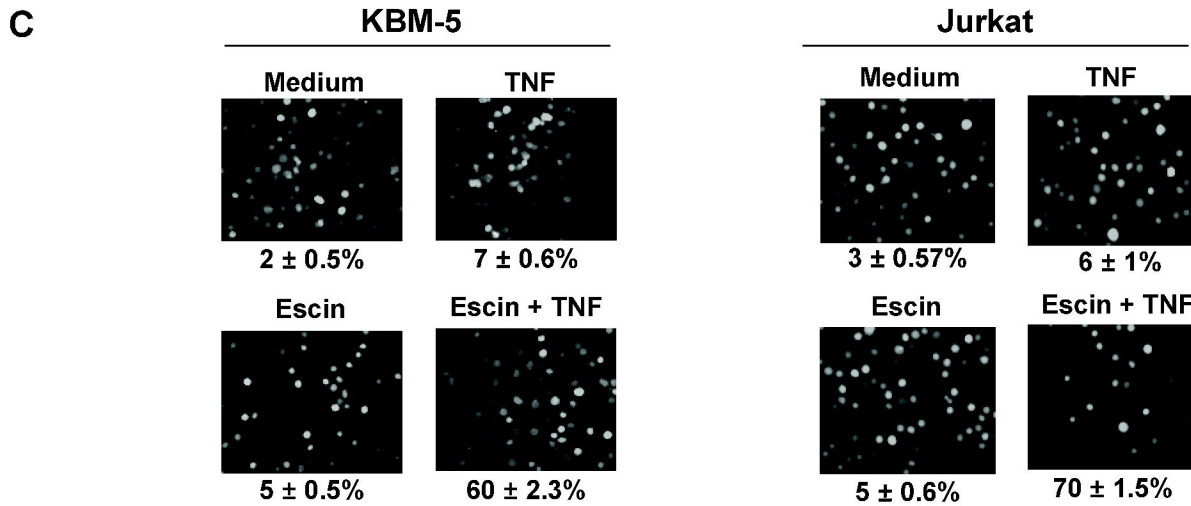
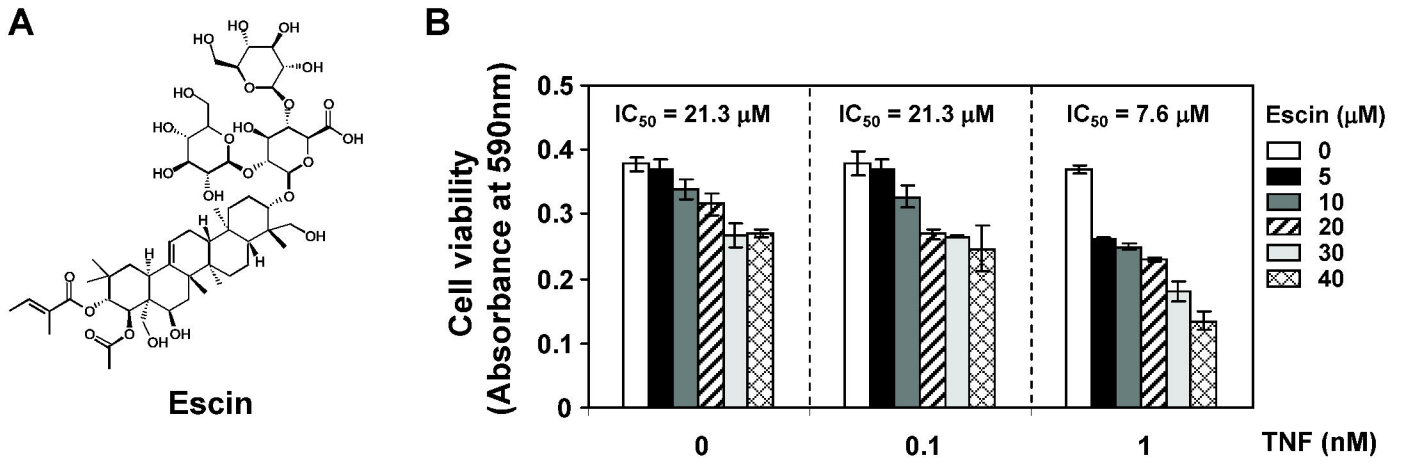
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were prepared and analyzed by Western blotting using p65 and phospho-specific p65 antibodies. For loading control of nuclear protein, the membrane was reblotted with either β -actin or anti-PARP antibody.

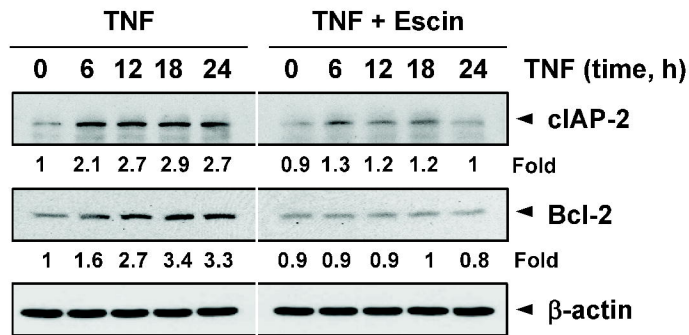
FIGURE 6: Effects of escin on NF- κ B-dependent reporter gene expression

A, Escin inhibits TNF-induced NF- κ B-dependent reporter gene (SEAP) expression.

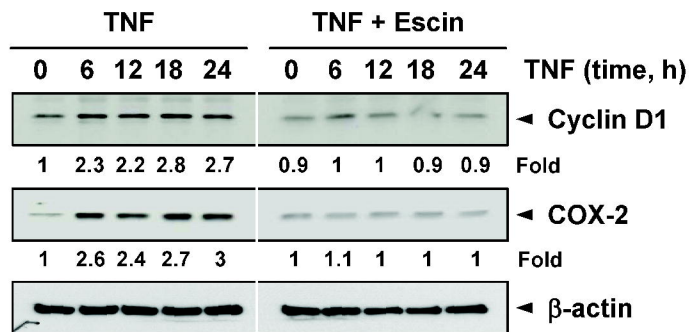
A293 cells (2.5×10^5 cells/mL) were transiently co-transfected with a NF- κ B-containing plasmid linked to the SEAP gene, after 24 h of transfection cells were treated with the indicated concentrations of escin for 2 h followed by 1nM of TNF for additional 24 h. Cell supernatants were collected and assayed for SEAP activity as described under "Materials and Methods". Results are expressed as fold activity over the activity of the vector control. **B**, Escin inhibited NF- κ B-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, IKK and p65. A293 cells were transiently transfected with the indicated plasmids along with a NF- κ B-containing plasmid linked to the SEAP gene and after 24 h cells were either treated or untreated with 30 μ M escin for 2 h. Where indicated, cells were exposed to 1 nM TNF for 12 h. Cell supernatants were assayed for SEAP activity as described under "Materials and Methods". Results are expressed as fold activity over the activity of the vector control. **C**, Whole cell extracts from transfected cells with various plasmids were prepared and analyzed by Western blotting using antibodies against TNFR1, TRADD, TRAF2, NIK, IKK- β , and p65. *DN*, dominant negative. All results shown are representative of two independent experiments.



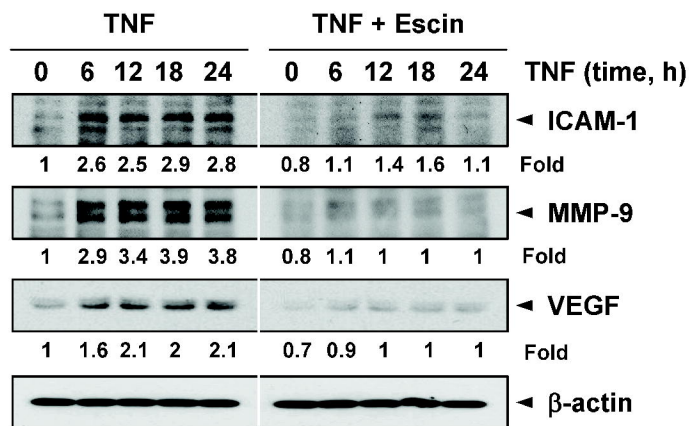
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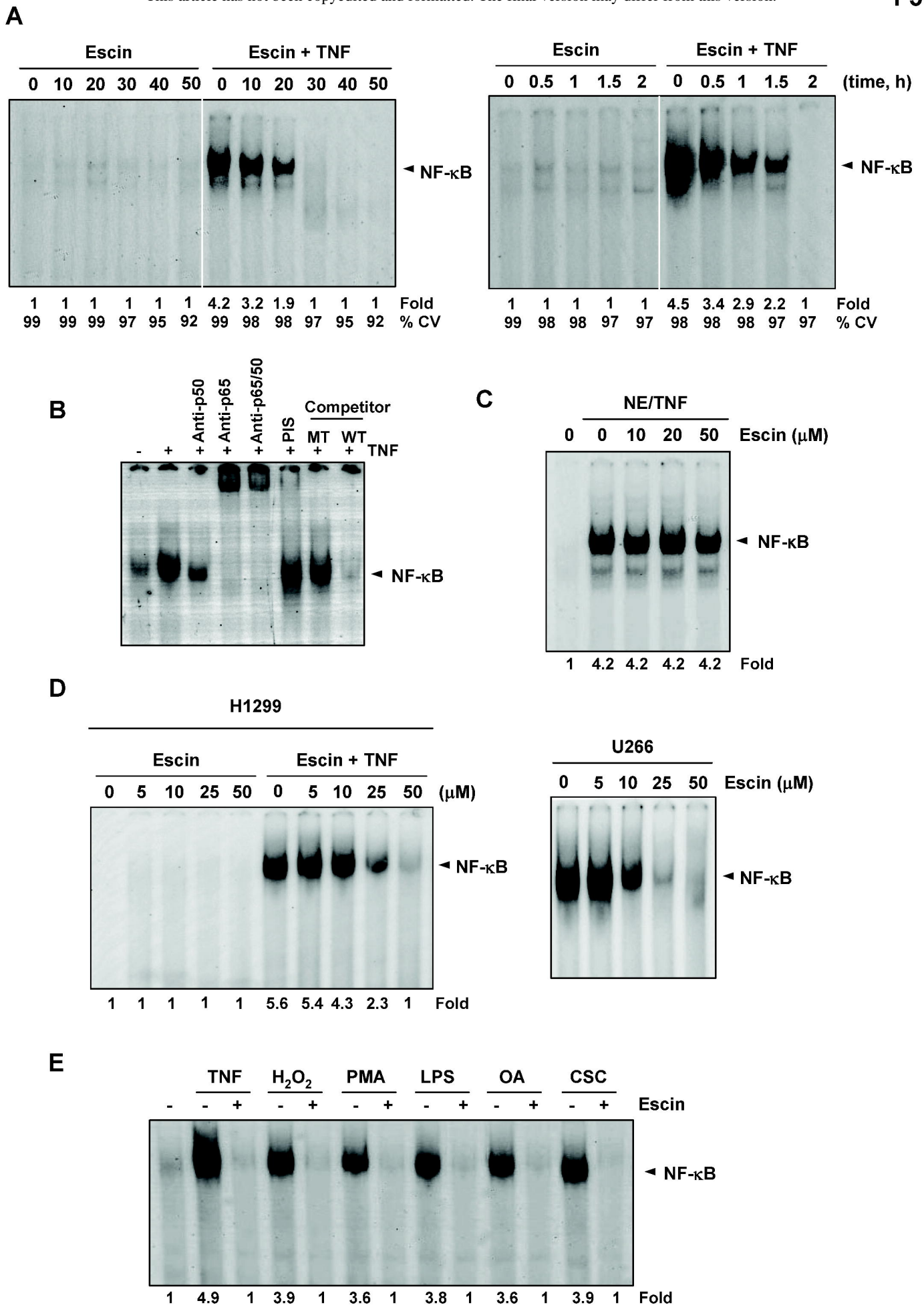


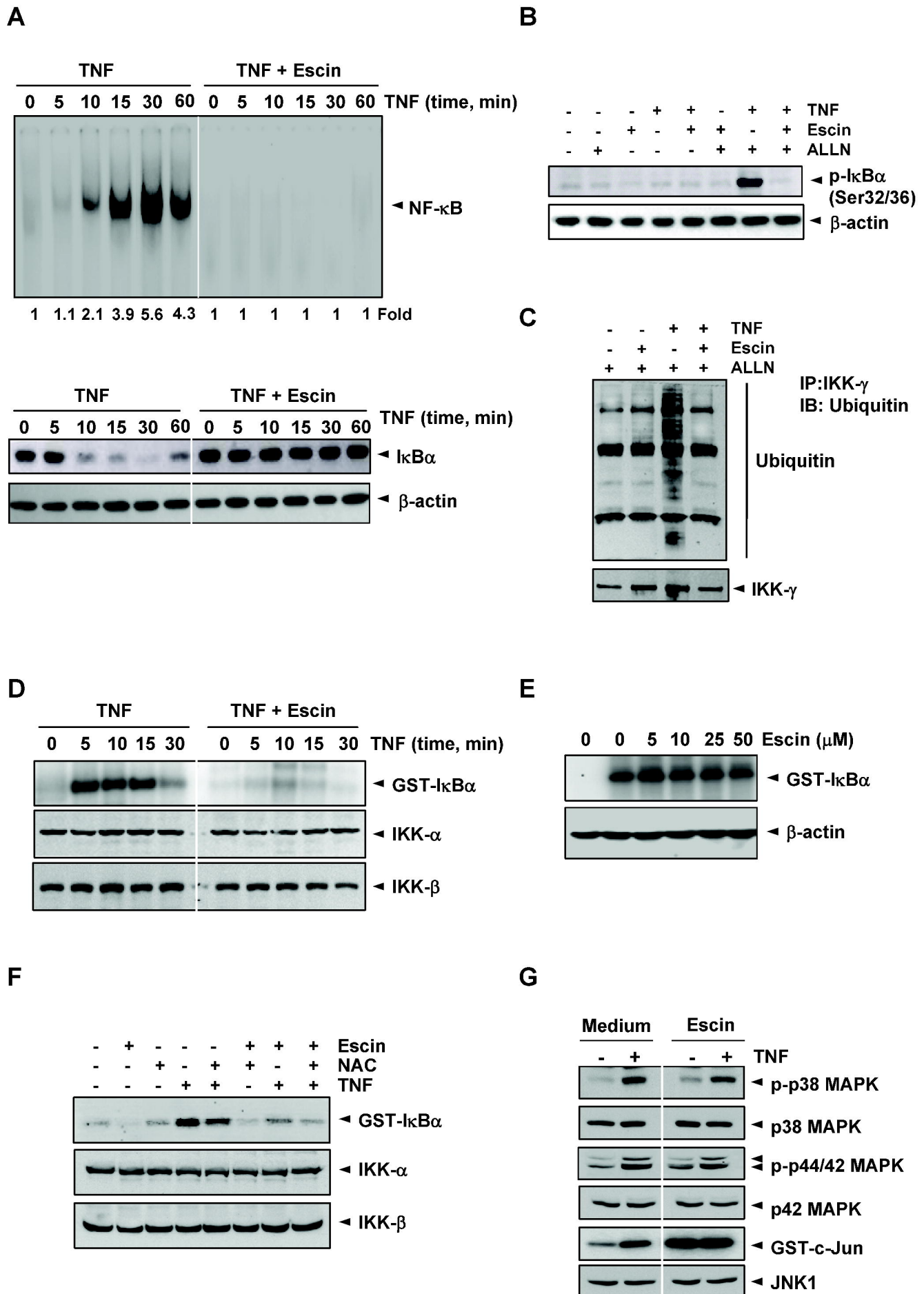
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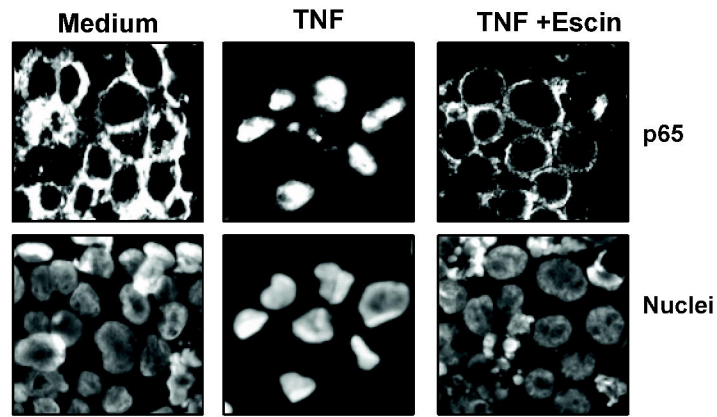
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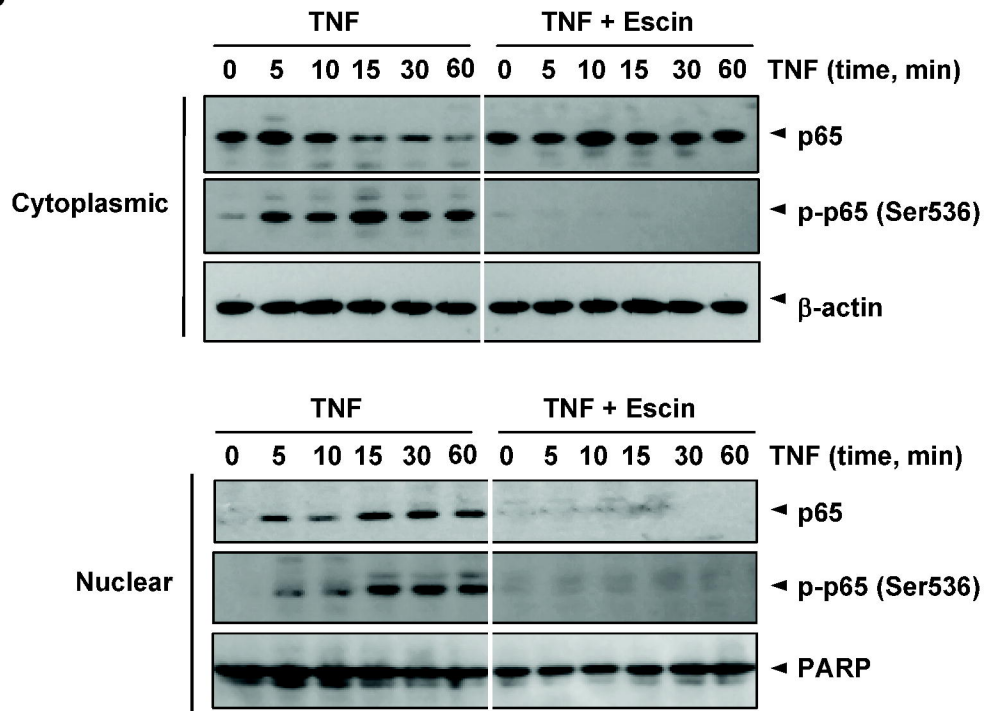




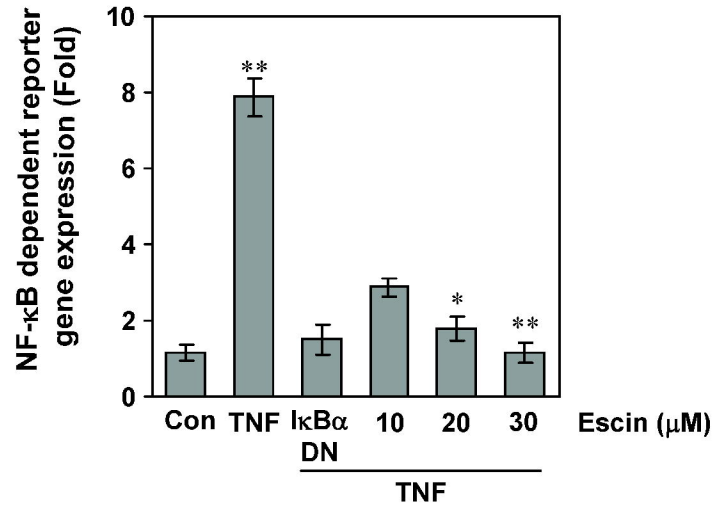
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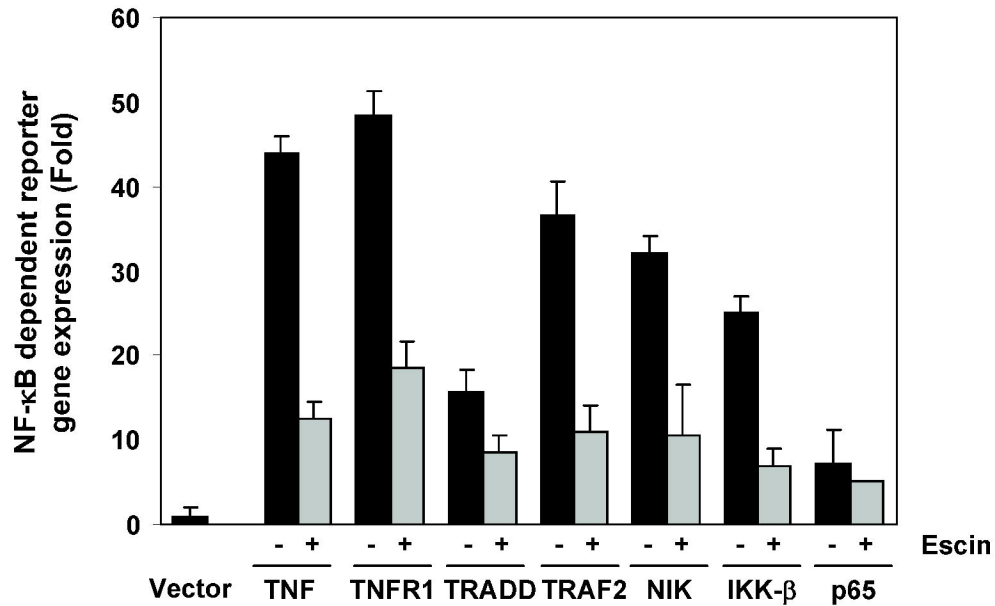
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